

Endothelin Is a Downstream Mediator of Profibrotic Responses to Transforming Growth Factor β in Human Lung Fibroblasts

Xu Shi-wen,¹ Laura Kennedy,² Elisabetta A. Renzoni,³ George Bou-Gharios,⁴
Roland M. du Bois,³ Carol M. Black,¹ Christopher P. Denton,¹
David J. Abraham,¹ and Andrew Leask²

Objective. Fibrosis is excessive scarring caused by the accumulation and contraction of extracellular matrix proteins and is a common end pathway in many chronic diseases, including scleroderma (systemic sclerosis [SSc]). Indeed, pulmonary fibrosis is a major cause of death in SSc. Transforming growth factor β (TGF β) induces endothelin 1 (ET-1) in human lung fibroblasts by a Smad-independent, JNK-dependent mechanism. The goal of this study was to assess whether ET-1 is a downstream mediator of the profibrotic effects of TGF β in lung fibroblasts.

Methods. We used a specific endothelin receptor

antagonist to determine whether ET-1 is a downstream mediator of TGF β responses in lung fibroblasts, using microarray technology, real-time polymerase chain reaction, and Western blot analyses.

Results. The ability of TGF β to induce the expression of a cohort of profibrotic genes, including type I collagen, fibronectin, and CCN2, and to contract a collagen gel matrix, depends on ET-1.

Conclusion. ET-1 contributes to the ability of TGF β to promote a profibrotic phenotype in human lung fibroblasts, consistent with the notion that endothelin receptor antagonism may be beneficial in controlling fibrogenic responses in lung fibroblasts.

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¹Xu Shi-wen, PhD, Carol M. Black, MD, FRCP, DBE, Christopher P. Denton, PhD, FRCP, David J. Abraham, PhD: Royal Free Hospital, University College London, London, UK; ²Laura Kennedy, BMSc, Andrew Leask, PhD: University of Western Ontario, London, Ontario, Canada; ³Elisabetta A. Renzoni, MD, Roland M. du Bois, MD: Royal Brompton Hospital, Imperial College of Science, Technology and Medicine, London, UK; ⁴George Bou-Gharios, PhD: Hammersmith Hospital, Imperial College of Medicine, London, UK.

Dr. Shi-wen, Ms Kennedy, and Dr. Renzoni contributed equally to this work.

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Address correspondence and reprint requests to Andrew Leask, PhD, CIHR Group in Skeletal Development and Remodeling, Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Dental Sciences Building, University of Western Ontario, London, Ontario N6A 5C1, Canada. E-mail: Andrew.Leask@schulich.uwo.ca.

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Tissue repair requires that new connective tissue be synthesized. Accordingly, fibroblasts must migrate into the wound and synthesize and remodel extracellular matrix (ECM). The specialized form of fibroblast that performs this function is the myofibroblast, so-called because it expresses a highly contractile form of actin, α -smooth muscle actin (α -SMA) (1). If the tissue repair program does not properly terminate, scarring results. Excessive scarring can cause pathologic fibrotic diseases, which can affect individual organs, such as in idiopathic pulmonary fibrosis or diabetic nephropathy, or it can be systemic, such as in scleroderma (systemic sclerosis [SSc]). Fibrotic diseases are a significant cause of mortality. Currently, there is no approved treatment for organ fibrosis.

Transforming growth factor β (TGF β) ligands promote ECM synthesis and remodeling, and myofibroblast differentiation (2). Exaggerated TGF β signaling in fibrotic fibroblasts contributes to particular features of their persistent fibrotic phenotype (3,4). However, TGF β also plays an important role in cell proliferation, lineage determination, cell motility, apoptosis, and modulation of immune function (2,5). Consequently, general

targeting of TGF β signaling to combat fibrotic diseases is therefore likely to be problematic due to the possibility of side effects; identification of downstream mediators that affect the profibrotic responses to TGF β in fibroblasts is therefore likely to yield more appropriate targets for antifibrotic drug intervention (6).

The vasoconstrictive peptide endothelin 1 (ET-1) also contributes to persistent fibrosis (7,8); increased ET-1 production by fibroblasts is a hallmark of fibrotic disease, including SSc (9,10). ET-1 signals through 2 receptors, ET_A and ET_B (11). ET-1 has profibrotic effects similar to those of TGF β , in that ET-1 causes fibroblasts to produce and contract ECM (7,12). TGF β induces ET-1 in normal and SSc lung fibroblasts through a Smad-independent, activin receptor-like kinase 5 (ALK-5)/JNK-dependent mechanism and an activator protein 1 (AP-1) site in the ET-1 promoter (8). Although these data suggest that ET-1 is a downstream mediator of TGF β in lung fibroblasts, this hypothesis has yet to be tested. Accordingly, in this study, we investigate whether ET-1 signaling contributes to fibrotic responses to TGF β in human lung fibroblasts.

MATERIALS AND METHODS

Subjects and cell culture. Fibroblasts were grown by explant culture from normal lungs not used for transplant. Fibroblasts were used at passage 3 (13,14). Informed consent and ethics committee approval were obtained. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μ g/ml streptomycin, and cultured in a humidified atmosphere of 5% CO₂ in air. Fibroblasts were subcultured at a dilution of 1:4 at confluence.

Western blot analysis. Normal lung fibroblasts were grown to confluence in DMEM with 10% fetal bovine serum and then serum starved in DMEM containing 0.5% bovine serum albumin (BSA) for 18 hours. After serum starvation, cells were incubated for an additional 1 hour in 10 μ M bosentan (Actelion, Allschwil, Switzerland) prior to the addition of TGF β 1 (4 ng/ml; R&D Systems, Minneapolis, MN). Cell layer lysates were examined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 12% polyacrylamide gels, and the separated proteins were transferred onto nitrocellulose membranes at 30V for 90 minutes. Membranes were blocked by incubation for 1 hour with 5% nonfat milk in phosphate buffered saline containing 0.2% Tween 20, and antigens were detected using specific antibodies. Cell layer lysates (25 μ g/sample) were probed using antibodies directed against CCN2, tissue inhibitor of metalloproteinases 3 (TIMP-3), GAPDH (Abcam, Cambridge, UK), fibronectin (Sigma, St. Louis, MO), and type I collagen (Biodesign International, Kennebunk, ME), followed by incubation with the appropriate horseradish peroxidase-conjugated bound secondary antibody (Jackson ImmunoRe-

search, Avondale, PA). The signal was detected using the enhanced chemiluminescence protocol (Amersham Biosciences, Piscataway, NJ), as described by the manufacturer.

Floating collagen gel cultures and quantitation of gel contraction. Experiments were performed essentially as previously described (7). Briefly, 24-well tissue culture plates were precoated with BSA. Trypsinized fibroblasts were suspended in MCDB medium and mixed with collagen solution (1 part HEPES, pH 8.0; 4 parts collagen [Vitrogen 100, 3 mg/ml; Cohesion Technologies, Palo Alto, CA]; and 5 parts MCDB [2 \times]), which yielded a final concentration of 80,000 cells/ml and 1.2 mg/ml of collagen. Collagen/cell suspension (1 ml) was added to each well. After polymerization, the gel was detached from the wells by adding 1 ml of MCDB medium. Contraction of the gel was quantified by loss of gel weight and decrease in gel diameter over a 24-hour period. For inhibition experiments, cells were preincubated in the presence of inhibitor for 30 minutes prior to initiation of the assay.

Expression profiling. Expression profiling was conducted as previously described (7,15,16). Lung fibroblasts were cultured until confluence, then serum starved for 18 hours. Fibroblasts were used at passage 3. Media were changed and cultured for an additional 1 hour in the presence or absence of bosentan prior to treatment with TGF β 1 (4 ng/ml for 6 hours). At the end of the treatment period, total RNA was harvested (TRIzol; Life Technologies, Gaithersburg, MD), quantified, and the integrity was verified by denaturing gel electrophoresis. Equal amounts of identically treated RNA were pooled into 2 separate reactions (containing RNA from 3 individuals each) and reverse transcribed (Life Technologies) into complementary DNA, which was then in vitro transcribed into biotinylated complementary RNA (cRNA). Target cRNA was fragmented and hybridized to a human genome U133A 2.0 array (Affymetrix, Santa Clara, CA) following a standard Affymetrix protocol. Hybridization of cRNA to Affymetrix U133A human gene chips, signal amplification, and data collection were performed using an Affymetrix fluidics station and chip reader. Chip files were scaled to an average intensity of 100 per gene and analyzed using Affymetrix version 5.0 (MAS5) comparison analysis software. Data were exported into Genespring (Silicon Graphics, Mountain View, CA) for further analysis.

Fold changes are presented in Supplementary Table 1 (available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>). Criteria indicated by microarray were used to determine robust changes in gene expression. Briefly, transcripts were defined as up-regulated in response to TGF β only when they were identified as "present" by the microarray detection algorithm, and were defined as significantly increased as determined by the Affymetrix change algorithm ($P < 0.01$). Of these, transcripts were defined as down-regulated in response to ET receptor antagonism when the fold change between treated and untreated samples was at least 2-fold.

Reverse transcriptase-polymerase chain reaction (PCR). Lung fibroblasts were serum starved for 18 hours and treated in the presence or absence of 10 μ M bosentan for 1 hour prior to treatment with or without TGF β 1 (4 ng/ml) for an additional 6 hours. Total RNA was isolated using TRIzol (Invitrogen, San Diego, CA), and further purified using

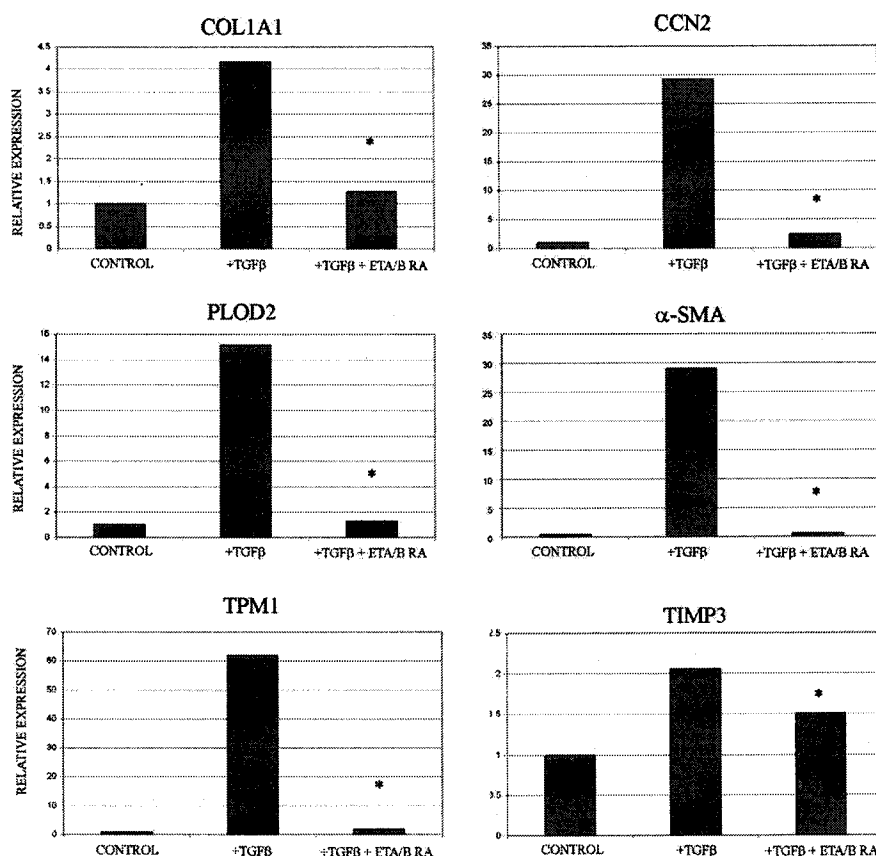


Figure 1. Effect of endothelin A/endothelin B (ET_A/ET_B) receptor inhibition on the ability of transforming growth factor β (TGF β) to induce profibrotic mRNA expression in lung fibroblasts. Normal human lung fibroblasts were cultured and treated with or without TGF β 1 for 6 hours, in the presence or absence of the ET_A/ET_B receptor antagonist (ETA/B RA) bosentan. RNA was harvested, and reverse transcriptase–polymerase chain reaction was used to detect mRNA for COL1A1, CCN2, PLOD2, α -smooth muscle actin (α -SMA), tropomyosin 1 (TPM1), and tissue inhibitor of metalloproteinases 3 (TIMP-3). As a control, 28S ribosomal RNA (rRNA) was amplified. Values are the mean of 3 replicates, relative to 28S rRNA expression values (set at 1). * = $P < 0.05$ versus treatment with TGF β 1 without ET_A/ET_B receptor antagonist.

RNeasy columns (Qiagen, Chatsworth, CA). Integrity of the RNA was verified by gel electrophoresis. Total RNA (25 ng) was reverse transcribed and amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems, Streetsville, Ontario, Canada) in a 15- μ l reaction volume containing 2 unlabeled primers and 6-carboxyfluorescein-labeled TaqMan minor groove binder probe sequences. Samples were combined with TaqMan One-Step Master Mix (Applied Biosystems). Amplified sequences were detected using the ABI Prism 7900 HT sequence detector (PerkinElmer Cetus, Norwalk, CT) according to the manufacturer's instructions. Triplicate samples were run, and expression values were standardized to values obtained with control 28S ribosomal RNA primers.

Statistical analysis. Data were analyzed using Student's paired t -test or using nonparametric tests, as appropriate. The probability values obtained are indicated in the text

and/or in the figure legends when statistically significant. Comparisons of collagen gel contraction were performed using Student's unpaired t -test. P values less than 0.05 were considered statistically significant.

RESULTS

ET-1 signaling and TGF β induction of a tissue remodeling phenotype. TGF β induces ET-1 expression in human lung fibroblasts through AP-1/JNK/ALK-5 (8). To evaluate the extent to which ET-1 contributes to the ability of TGF β to induce a fibrotic phenotype in lung fibroblasts, we cultured normal lung fibroblasts until 80% confluence. Cells were serum starved for 18 hours, pretreated for 1 hour with or without the dual ET_A/ET_B

receptor antagonist bosentan (10 μ M), and then further incubated in the presence or absence of TGF β 1 (4 ng/ml for 6 hours). Bosentan was chosen for our experiments to ensure that all ET-dependent genes were detected. Total RNA was prepared from these cells, reverse transcribed, and applied to Affymetrix human genome U133A 2.0 arrays. Cluster analysis revealed that the TGF β induction of 109 transcripts involved with tissue remodeling was reduced by ET receptor antagonism (Supplementary Table 1). Included in these genes were transcripts encoding procontractile genes, such as tropomyosin 1 and α -SMA; adhesive genes, such as CCN2 (connective tissue growth factor) (17,18); and those promoting matrix accumulation, such as type I collagen and TIMP-3, as well as the ECM crosslinking gene PLOD2 (19). Intriguingly, ET_A/ET_B receptor antagonism did not significantly affect basal messenger RNA (mRNA) expression in normal fibroblasts (supplemental Table 1). These data suggest that ET-1 acts downstream of TGF β to promote a fibrotic phenotype in lung fibroblasts.

Verification of gene profiling data by real-time PCR and Western blot analysis. We then sought to confirm our gene profiling data showing that ET-1 was required for lung fibroblasts to respond to TGF β by inducing the expression of matrix and proadhesive proteins. Therefore, real-time PCR analysis was used to determine whether antagonizing ET-1 signaling through the ET_A/ET_B receptors could block the TGF β induction of profibrotic genes. Lung fibroblasts were serum starved for 18 hours, and treated for 60 minutes with or without bosentan (10 μ M). Cells were treated for an

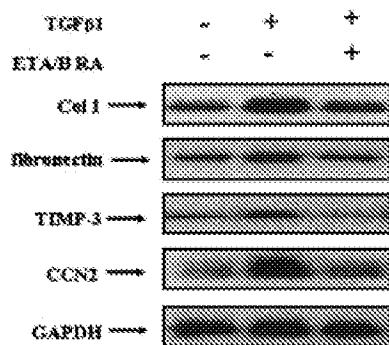


Figure 2. Demonstration by Western blot analysis that overexpression of type I collagen, fibronectin, TIMP-3, and CCN2 by fibrotic fibroblasts is blocked by ET_A/ET_B receptor inhibition. Cell lysates (25 μ g) from lung fibroblasts treated with or without TGF β 1 for 24 hours, in the presence or absence of bosentan, were subjected to Western blot analysis using anti-type I collagen, antifibronectin, anti-TIMP-3, and anti-CCN2. See Figure 1 for definitions.

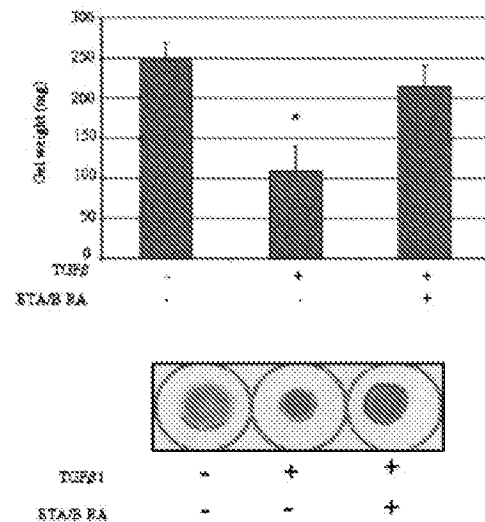


Figure 3. Demonstration by floating gel contraction assay that TGF β induces collagen gel contraction in fibroblasts. Lung fibroblasts were placed within collagen gel lattices. After polymerization, lattices were detached from tissue culture plates and treated with or without TGF β 1 (4 ng/ml) for 24 hours in the presence or absence of bosentan. Contraction was monitored by measuring gel weight. **Top**, Mean and SD gel weight, as a measure of contraction. * = $P < 0.05$ versus untreated control cells. **Bottom**, Photograph showing the contraction obtained in each gel. See Figure 1 for definitions.

additional 6 hours with and without TGF β 1 (4 ng/ml). We then harvested mRNA from these cells and used real-time PCR analysis to confirm the gene profiling data showing that the ability of TGF β to induce the expression of tropomyosin 1, α -SMA, CCN2, type I collagen, TIMP-3, and PLOD2 mRNA was dependent on ET signaling (Figure 1). It is interesting to note that the induction of TIMP-3 was less than 2-fold, by real-time PCR analysis. Second, Western blot analysis was used to show that bosentan could block the ability of TGF β 1 (4 ng/ml for 24 hours) to induce type I collagen and CCN2 protein (Figure 2). Therefore, in lung fibroblasts, ET-1 was acting downstream of TGF β to promote the expression of profibrotic genes.

Ability of TGF β to promote ECM contraction decreased by bosentan. To provide a functional context for the microarray, real-time PCR, and Western blot analysis data, and to further address the potential contribution of endogenous ET-1 signaling (via the ET_A/ET_B receptors) to the profibrotic responses of lung fibroblasts to TGF β , we assessed the abilities of ET_A/ET_B receptor antagonism to lessen the ability of TGF β to induce pulmonary fibroblasts to contract a collagen gel matrix. Lung fibroblasts were combined with collagen and allowed to polymerize in a 6-well plate for 1

hour in the presence or absence of bosentan. The polymerized gel was detached from the plate and incubated for an additional 24 hours with or without TGF β 1 (4 ng/ml). As expected, TGF β induced fibroblasts to contract a collagen gel matrix (Figure 3). However, the ability of TGF β to promote collagen gel contraction was significantly reduced if the cells were pretreated with bosentan (Figure 3). Collectively, our results showed that endogenous ET-1 is essential for TGF β induction of a fibrotic phenotype in lung fibroblasts.

DISCUSSION

In this study, we examined the signaling events downstream of TGF β that result in an ECM remodeling phenotype. In vitro and in vivo studies have repeatedly demonstrated that TGF β enhances ECM production and contraction in fibroblasts, leading to granulation tissue deposition and scarring (20,21). Most previous studies have focused on immediate or early responses to TGF β resulting in the production and remodeling of ECM in fibroblasts (22,23). However, due to the pleiotropic nature of TGF β , it would be beneficial in promoting and controlling the wound healing and scarring while leaving other effects of TGF β unaltered (2,6). Moreover, because fibrosis is a chronic disease, identifying proteins that act downstream of TGF β may reveal likely candidates that mediate persistent fibrotic responses to TGF β and, hence, reveal more appropriate targets for drug intervention in chronic fibrotic disease.

In addition to TGF β , ET-1 (4,7) also promotes fibrotic responses in fibroblasts. In this study, we investigated the ability of bosentan, a dual ET $_A$ /ET $_B$ receptor antagonist (11), to prevent the expression of profibrotic genes in response to TGF β in lung fibroblasts. Our data extend previous findings that TGF β induces ET-1 in lung fibroblasts through an AP-1 site in the ET promoter and that ET-1 antagonism alleviates that persistent fibrotic phenotype of SSc fibroblasts (7,8). Here, we show that ET-1 signaling in lung fibroblasts contributes to the ability of TGF β to induce expression of key profibrotic markers, including α -SMA, CCN2, and type I collagen. In addition, we show that the ability of TGF β to induce a procontractile phenotype in pulmonary fibrotic fibroblasts depends on ET-1. Collectively, these data, combined with our previous results showing that an autocrine ET-1 loop is responsible for the persistent fibrotic phenotype of SSc fibroblasts (7,8), support the notion that ET-1 makes a significant contribution to fibrogenesis. Consequently, ET $_A$ /ET $_B$ receptor antago-

nism may be a useful method by which to alleviate fibrosis.

In conclusion, we provide evidence that ET-1 is a downstream mediator of TGF β responses in lung fibroblasts and provide further support for the potential use of ET receptor antagonists in treating pulmonary fibrosis.

AUTHOR CONTRIBUTIONS

Dr. Leask had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Shi-wen, Kennedy, Renzoni, Black, Denton, Abraham, Leask.

Acquisition of data. Shi-wen, Kennedy, Renzoni, Bou-Gharios.

Analysis and interpretation of data. Shi-wen, Kennedy, Renzoni, du Bois, Denton, Abraham, Leask.

Manuscript preparation. Shi-wen, Renzoni, du Bois, Black, Denton, Abraham, Leask.

Statistical analysis. Shi-wen, Kennedy, Renzoni.

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